


Title	High-throughput screening in colorectal cancer tissue-originated spheroids
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## ORIGINAL ARTICLE

# High-throughput screening in colorectal cancer tissue-originated spheroids

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Patient-derived cancer organoid culture is an important live material that reflects clinical heterogeneity. However, the limited amount of organoids available for each case as well as the considerable amount of time and cost to expand in vitro makes it impractical to perform high-throughput drug screening using organoid cultures from multiple patients. Here, we report an advanced system for the high-throughput screening of 2427 drugs using the cancer tissue-originated spheroid (CTOS) method. In this system, we apply the CTOS method in an ex vivo platform from xenograft tumors, using machines to handle CTOS and reagents, and testing a CTOS reference panel of multiple CTOS lines for the hit drugs. CTOS passages in xenograft tumors resulted in minimal changes of morphological and genomic status, and xenograft tumor generation efficiently expanded the number of CTOS to evaluate multiple drugs. Our panel of colorectal cancer CTOS lines exhibited diverse sensitivities to the hit compounds, demonstrating the usefulness of this system for investigating highly heterogeneous disease.

## KEYWORDS

3D culture, cancer, heterogeneity, high throughput screening, organoid

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## 1 | INTRODUCTION

Inter-patient variations in sensitivity to therapeutics are a challenging issue in clinical practice, making it increasingly important to obtain tumor samples from individual cancer patients for both development of therapeutics and personalized medicine. To determine the portion of the population in which a drug will be effective and to identify biomarkers to demarcate potential responders, it is essential to develop systems in which drugs can be tested on materials retaining the heterogeneous characteristics of the original disease.<sup>1-3</sup>

To assess the responses of cancer cells to various stimuli, including anti-cancer drugs, culture of cancer cells is required. Established cancer cell lines have historically been the pillar of such in vitro models. The simplicity and reproducibility of cancer cell lines enables drug testing and screening.<sup>4,5</sup> However, prolonged in vitro culture of cancer cells promotes cell conversion, probably by selection or accumulation of genetic alterations, which is a caveat for the development of personalized therapeutic approaches and of new drugs.<sup>5</sup>

Over the past decade, primary culture has been significantly improved by overcoming complicated procedures, poor reproducibility and uncontrolled co-existence of various cell types.<sup>1,6,7</sup> Modern cultures are expected to retain the characteristics of parental patient tumors, including drug sensitivity. One such approach is our recently developed method to efficiently prepare and culture organoids from primary tissues of colorectal cancers (CRC): the cancer tissue-originated spheroid (CTOS) method.<sup>8</sup> The principle behind the CTOS method is the maintenance of cell-cell contact throughout the preparation and culture process. This method provides the advantage of efficiently, rapidly and simply preparing pure and stable cancer cells as clusters. Some CTOS can form xenograft tumors in immuno-deficient mice, with morphology resembling that of the originated patient tumor.<sup>8</sup> Importantly, CTOS are simple to prepare, especially from xenograft tumors, enabling their reproducible application in multiple experiments.<sup>9-12</sup> We previously demonstrated that CTOS exhibit heterogeneous responses to the tested drugs,<sup>9,12</sup> which is critical in models for developing therapeutics and personalized medicine.

We have used the CTOS method to perform several small-scale drug screening trials, testing approximately 100 drugs with CTOS from CRC and endometrial cancer.<sup>12-14</sup> However, with manual experiments, it is practically impossible to perform high-throughput (HT) screening to test thousands of drugs. Because cell-cell contact is retained throughout the procedure in the CTOS method, the well-developed screening method for 3D culture of established cell lines cannot be simply applied. In our present report, we describe a highly improved system to achieve HT screening of 2427 drugs. We confirmed that CTOS passages in xenograft tumors resulted in minimal changes in morphological and genomic status, and that xenograft tumor generation efficiently expanded the number of CTOS, enabling high throughput screening. Our panel of 30 CRC CTOS lines exhibited diverse sensitivities to the hit compounds, demonstrating the usefulness of this system for investigating highly heterogeneous cancers.

## 2 | MATERIALS AND METHODS

### 2.1 | Generation of cancer-tissue originated spheroid lines and patient-derived xenograft lines

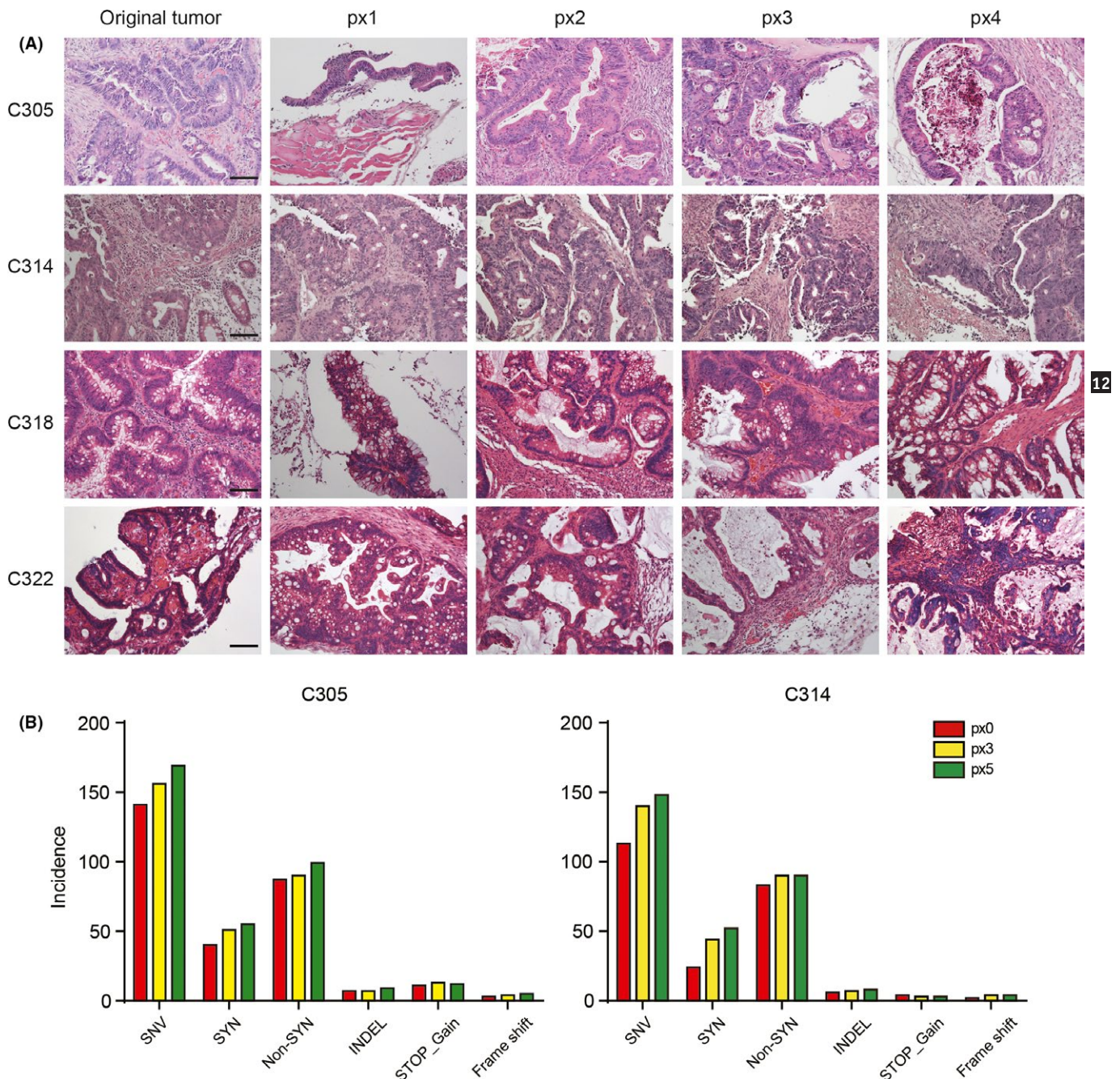
Surgical specimens, endoscopic biopsy and pleural effusion samples from CRC and lung cancer patients were obtained from the Osaka International Cancer Institute (formerly the Osaka Medical Center for Cancer and Cardiovascular Diseases) with the patients' informed consent and permission of the Institutional Review Board. Tumor tissue samples for patient-derived xenografts (PDX) were obtained from Osaka Police Hospital, with the patients' informed consent and approval from the National Institutes of Biomedical Innovation, Health and Nutrition. Animal studies were performed in compliance with the guidelines of the institutional animal study committee of the Osaka International Cancer Institute and the National Institutes of Biomedical Innovation, Health and Nutrition.

Primary CTOS were prepared from patient tumor samples, and inoculated into non-obese diabetic/severe combined immunodeficient (NOD/Scid) mice (CLEA Japan, Tokyo, Japan). CTOS were also prepared from the xenograft tumors, and were freeze-stocked or inoculated into NOD/Scid mice. CTOS lines were generated fulfilling the following criteria: at least 2 in vivo passages, and sufficient freeze-stocked CTOS to generate another passage of xenograft tumors and to reproduce the experiments. Tables S1, S2 and S3 present details regarding the 32 CTOS lines in the CRC panel. The PDX lines were established as previously described.<sup>15</sup> Table S3 presents details regarding the original tumors for the 3 PDX lines. Formalin-fixed paraffin-embedded tissues were used for morphological analysis of original CTOS-derived xenograft and patient-derived xenograft tumors.

### 2.2 | Cancer tissue-originated spheroid preparation

For both surgical specimens and xenografts, CTOS were prepared as previously described with slight modifications.<sup>8</sup> Briefly, tumors were mechanically minced and incubated for 90 minutes at 37°C with continuous stirring, in DMEM/Ham's F12 medium (Wako Pure Chemical Industries, Osaka, Japan) containing Liberase DH (Roche, Basel, Switzerland) at a final concentration of .28 units/mL. DNase I (Roche) was added at 10 µg/mL, followed by an additional 15 minutes of incubation. The digestion solution was serially strained using mesh filters of 500, 250, 100 and 40 µm (BD Falcon, Franklin Lakes, NJ, USA). Fractions were recovered between 100-250 µm (Fr.100-250) and 40-100 µm (Fr.40-100). Fr.40-100 samples were cultured for 24-48 hours at 37°C under 5% CO<sub>2</sub>, 20% O<sub>2</sub>, in STEMPRO hESC SFM (Invitrogen, Carlsbad, CA, USA) to form CTOS. Fr.100-250 samples were mechanically disrupted by raising and lowering the plunger several times using a 27-gauge needle, and then cultured as described for Fr.40-100. To generate xenograft tumors of CTOS lines, freeze-stocked CTOS<sup>12</sup> were thawed, and cultured for 7 days in STEMPRO hESC SFM. We then suspended 2000 CTOS in a 1:1 mixture of medium and Matrigel GFR (Corning, Corning, NY, USA), and subcutaneously injected 1000 CTOS each into 2 sites of dorsal skin in NOD/scid mice.





**FIGURE 1** Morphological changes and genomic alterations during the passage of cancer tissue-originated spheroid (CTOS) lines. A, Images show hematoxylin and eosin staining of original tumors and xenograft tumors at different passage numbers. Passage number is expressed as px n. Representative 4 lines of colorectal cancer are shown. Scale bar, 100  $\mu$ m. B, Incidence of various genome alterations in CTOS lines of colorectal cancer at different passage numbers. INDEL, insertion or deletion; non-SYN, non-synonymous; SNV, single-nucleotide variation; SYN, synonymous

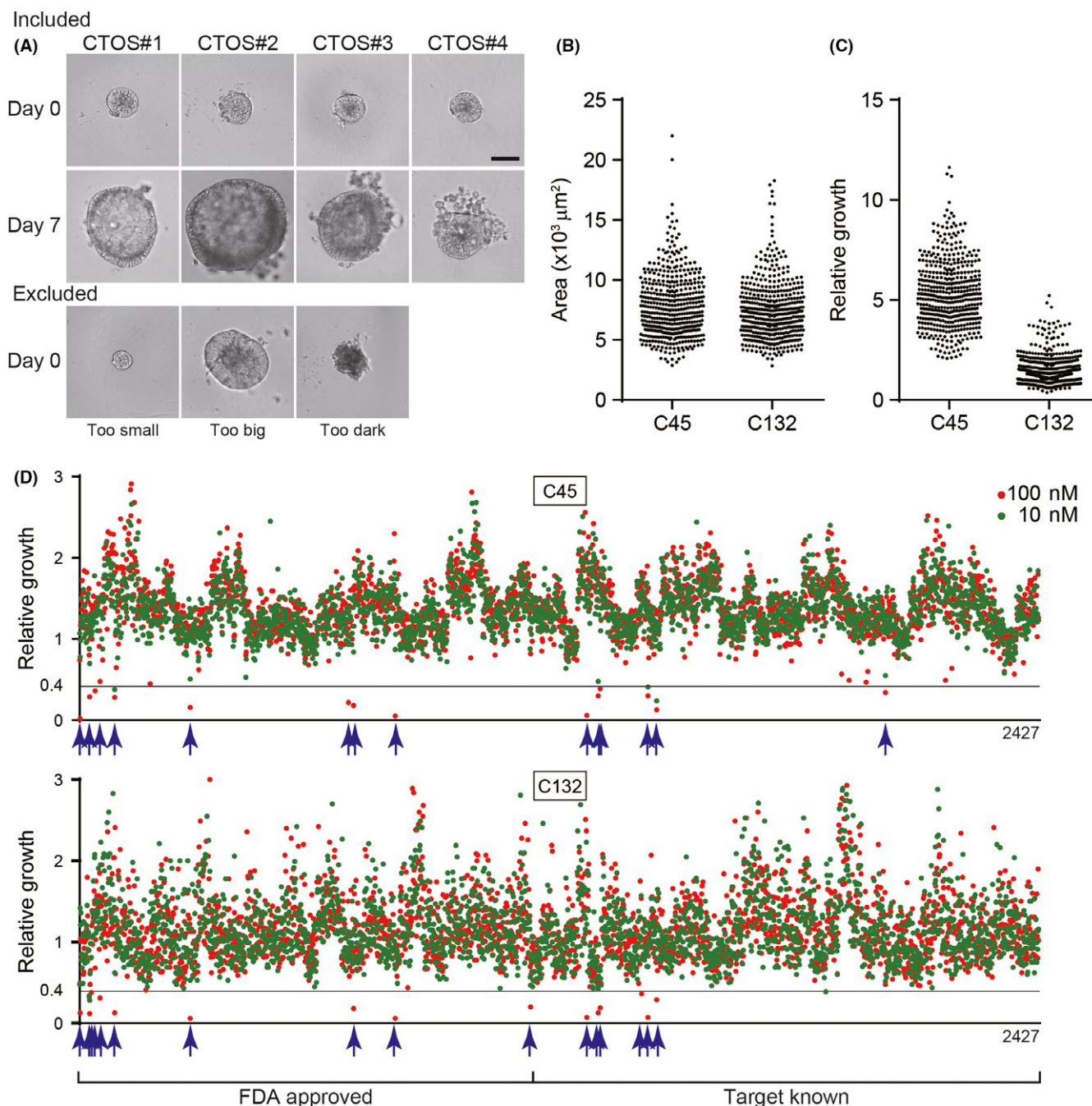
### 2.3 | Library preparation and sequencing for exome analysis

In preparation for exome sequencing analyses, extracted DNA samples from original tumors, normal peripheral blood cells, xenografts and 2D cultured cells were subjected to shearing, end repair, phosphorylation and ligation of barcoded adaptors. Next, the DNA underwent hybrid capture using SureSelect Human All Exon V5 (Agilent Technologies,

Santa Clara, CA, USA). Captured DNA was multiplexed and sequenced on a HiSeq 2500 or HiSeq 2000 system (Illumina, San Diego, CA, USA) with a median coverage of 245–343 per tumor, 104–211 per normal.

### 2.4 | Bioinformatics analysis of sequencing data

Sequenced reads were aligned with Burrows-Wheeler Aligner (BWA) ver. 0.7.12 to reference the human genome (hg19) or mouse genome



**FIGURE 2** High-throughput screening with colorectal cancer (CRC) cancer tissue-originated spheroid (CTOS) lines. A, Phase-contrast images of examples of C45 CTOS that were selected (upper panels) and excluded (bottom panels) for screening. Images after 7 d of culture are shown for each selected CTOS (middle panels). Reasons for exclusion were as follows: too small, too big and too dark, as shown from left to right (lower panels). Scale bar, 100  $\mu\text{m}$ . B, Areas of CTOS selected for screening. C, Relative growth of CTOS shown in (B) after 7 d of culture. D, High-throughput screening of 2427 drugs using 2 CTOS lines (C45 and C132) with 2 drug doses: 10 nmol/L (green dots) and 100 nmol/L (red dots). Arrows indicate the drugs that inhibited CTOS viability to 40% of control at 100 nmol/L

(mm9).<sup>16</sup> The reads were excluded if they appeared to be derived from mouse cells based on the following criteria: reads were properly paired when aligned to the mouse genome but not when aligned to the human genome, or reads were more similar to the mouse genome than the human genome in the match length. GenomeAnalysisTK (GATK) ver. 3.4-46 was used to recalibrate the variant quality score and to perform local realignment.<sup>17</sup> Somatic SNV were called using VarScan

ver.2.3.7,<sup>18</sup> MuTect ver. 1.1.5<sup>19</sup> and Karkinos ver. 3.0.22 (H. Ueda, unpublished). Somatic indels were detected using VarScan ver. 2.3.7, SomaticIndelDetector ver.2.3-9,<sup>17</sup> and Karkinos ver. 2.0.1. Somatic SNV and indels were considered genuine mutations when detected with at least 2 of the 3 callers. Somatic CNV were detected using EXCAVATOR ver. 2.2.<sup>20</sup> The sequence data are available in the NBDC Human Database under the accession number JGAS00000000089.



**TABLE 1** List of hit compounds from the screening

			Inhibition ratio		FDA approved
			C45	C132	
Compounds			Dose(nmol/L)		
C45/C132 both					
1	Bortezomib	10	0.78	1.42	Yes
		100	0.02	0.13	
2	Gemcitabine	10	0.70	0.28	Yes
		100	0.29	0.12	
3	Camptothecin	10	0.38	0.62	No
		100	0.28	0.13	
4	Cabazitaxel	10	0.84	0.75	Yes
		100	0.18	0.18	
5	Brefeldin A	10	1.55	1.22	No
		100	0.06	0.07	
6	CB 1954	10	1.80	0.65	No
		100	0.39	0.19	
7	Diphenyleneiodonium chloride	10	0.24	0.79	No
		100	0.13	0.29	
8	Ouabain	10	0.87	0.67	No
		100	0.05	0.06	
C45 alone					
9	Cladribine	10	1.29	1.39	Yes
		100	0.36	0.68	
10	Carfilzomib	10	0.93	1.13	Yes
		100	0.22	0.88	
C132 alone					
11	Docetaxel	10	0.88	0.34	Yes
		100	0.80	0.32	
12	Valproate	10	1.03	0.81	Yes
		100	1.31	0.38	
13	Clofarabine	10	1.48	1.51	Yes
		100	0.48	0.31	
14	PD-180970	10	1.13	0.47	No
		100	0.82	0.20	
15	CGS-15943	10	1.52	0.94	No
		100	1.48	0.36	

Inhibition ratios (see material and method) of <0.4 are indicated in red letters.

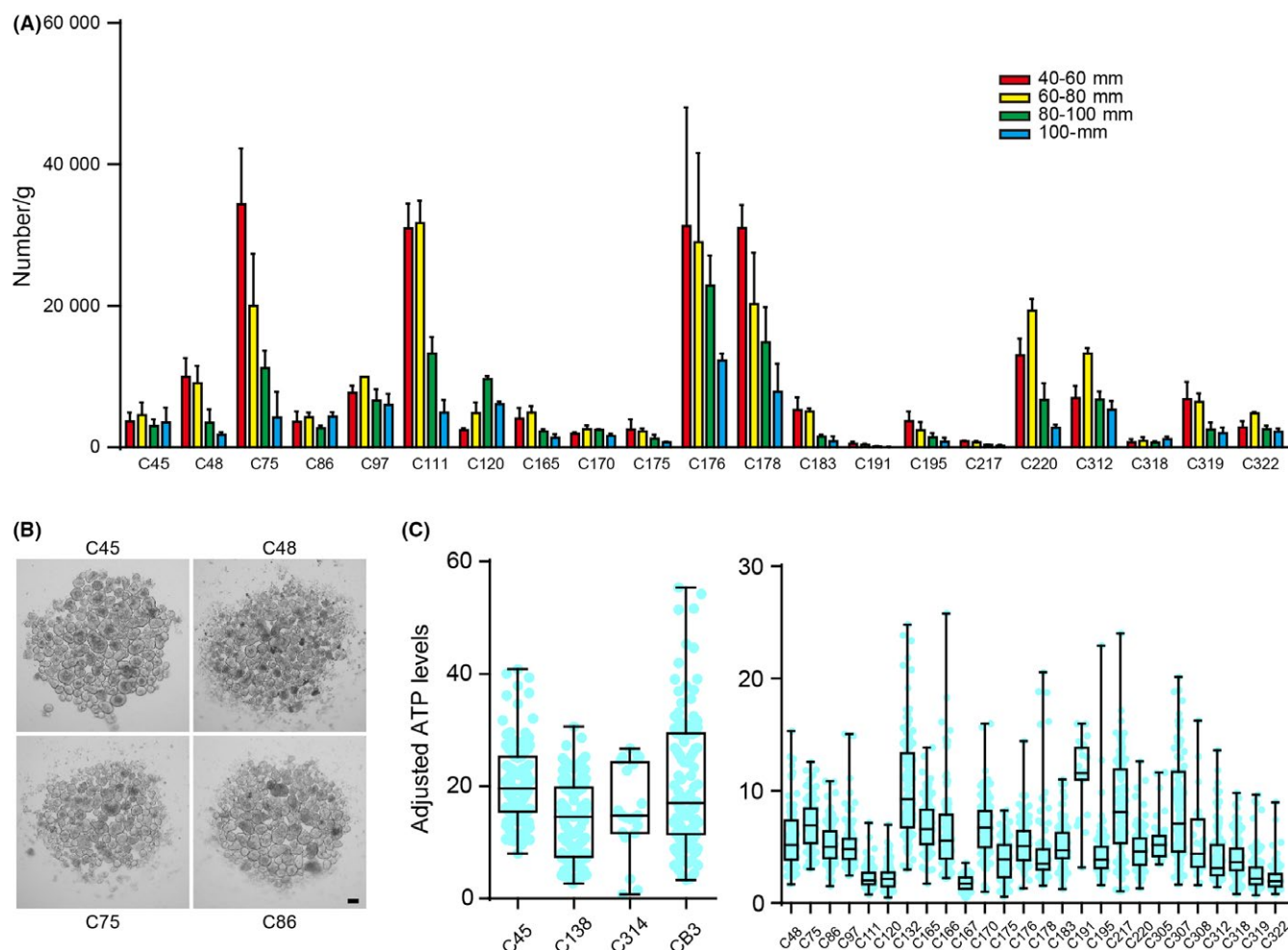
## 2.5 | Assay plate preparation

Cancer tissue-originated spheroids were prepared as described above. After culturing for 24 hours, the CTOS were separated into 2 fractions by serially passing through 100- $\mu$ m and 70- $\mu$ m cell strainers (Corning): 70-100  $\mu$ m (Fr.70-100) and >100  $\mu$ m (Fr. > 100). Fr.70-100 was directly selected and plated. Fr. > 100  $\mu$ m was mechanically disrupted, cultured for 1 day, and then selected and plated. An automated pipetting system (P5073; Eppendorf, Hamburg, Germany) was used to dispense 36  $\mu$ L of STEMPRO hESC SFM into 384-well plates (PrimeSurface, Sumitomo Bakelite, Tokyo, Japan). Then, the 384-well plate was seeded with 1 CTOS per well using an automatic

spheroid handler (CELL HANDLER <https://global.yamaha-motor.com/business/hc/index.html>; Yamaha Motor, Iwata, Japan). The CTOS were selected according to size and appearance (see text for details), using images acquired by the internally equipped CCD camera in the spheroid handler. The area of each CTOS was measured using the pictures taken after plating.

## 2.6 | Drug panel for high-throughput screening

The library included 2427 drugs (Table S4), including FDA-approved drugs and small molecules with known targets, which were provided by Platform Project for Supporting Drug Discovery and Life



**FIGURE 3** Colorectal cancer (CRC) cancer tissue-originated spheroid (CTOS) panel for validation of hit drugs. A, CTOS yield from xenograft tumors, showing the results from 21 CTOS lines. B, Four examples of the images used to count CTOS. Scale bar, 100  $\mu$ m. C, ATP levels at day 7, adjusted by the area on day 0. Each dot indicates the value for each single CTOS. Four lines with fast growth are shown on the left graph, and the remaining 26 lines are shown on the right

Science Research (Basis for Supporting Innovative Drug Discovery and Life Science Research [BINDS]). Each drug was diluted with DMSO and dispensed into 384-well plates at a final concentration of 10 or 100 nmol/L. Six wells were used for each concentration of each drug. Control wells (DMSO-treated and CTOS-free wells) were placed in every plate, with at least 6 wells for each control.

## 2.7 | Cancer tissue-originated spheroid viability assay

After addition of the drugs, CTOS were cultured in 5% CO<sub>2</sub> at 37°C for 7 days. Intracellular ATP was measured using the ATPlite 1step kit (PerkinElmer, Waltham, MA, USA). Chemiluminescence was detected by Alpha Plate (PerkinElmer), and the value was adjusted by CTOS area before drug addition at 0, 1, 3, 10, 30, 100, 300 and 1000 nmol/L. Alternatively, for the assay on PDX-derived CTOS (KC478, KC4876 and KC526), drugs were added at 0, 1, 10, 100 and 1000 nmol/L. Relative growth was calculated by dividing the area by that of the vehicle-treated control. The half-maximal (50%)

inhibitory concentration value (IC<sub>50</sub>) was calculated with GraphPad Prism 4 software (GraphPad, San Diego, CA, USA), using the sigmoidal dose-response function.

## 2.8 | Determination of drug efficacy against monolayer cell lines

The drug efficacy against established cell lines in monolayer culture was assessed based on total cellular protein changes after 48 hours of drug treatment in five 10-fold serial dilutions (10<sup>-4</sup>, 10<sup>-5</sup>, 10<sup>-6</sup>, 10<sup>-7</sup> and 10<sup>-8</sup> mol/L, unless otherwise specified), measured by sulforhodamine B assay. Assays were performed in duplicate, and the half-maximal (50%) growth inhibitory concentration value (GI<sub>50</sub>) was calculated as previously described.<sup>21,22</sup>

## 2.9 | Clustering analysis

The IC<sub>50</sub>/GI<sub>50</sub> values of a drug across the examined cells (CTOS or cell lines) were log-transformed and centered by subtracting the mean

log-transformed values. These values represent the drug's efficacy profile. Next, the drugs were clustered by efficacy profiles, based on correlation coefficients with other drugs (average-linkage clustered with Pearson's correlation metric). Similarly, cells were clustered according to their sensitivity profiles across the examined drugs, based on correlation coefficients with other cells. Cluster and Treeview software (Eisen Laboratory, Stanford University, CA, USA) were used for cluster analyses, visualization of clustering results by tree structure, and visualization of relative drug efficacy by heat map.

## 2.10 | Statistical analysis

Correlations were analyzed by Pearson's correlation using GraphPad Prism 6 software (San Diego, CA, USA). A *P*-value of <0.05 was considered significant.

## 3 | RESULTS

### 3.1 | Generating cancer tissue-originated spheroid-derived xenografts without remarkable alteration of histological and genetic profiles

To perform high-throughput (HT) screening on patient-derived materials and to evaluate diversity among them, we established CRC CTOS lines via *in vivo* passaging. CTOS were prepared from xenograft tumors, transplanted to other mice, and freeze-stocked. When a CTOS successfully generated xenograft tumors in over 2 passages, and left sufficient freeze stocks, it was deemed a successfully established CTOS line.

We next evaluated how well CRC CTOS retained characteristics of their originated patient tumors after *in vivo* passages. Established cell lines reportedly lose characteristics of the original tumors,<sup>5</sup> accumulating mutations and losing morphological features even in xenograft tumors.<sup>8</sup> We compared histology between xenograft tumors at different passages and the original patient tumors (Figure 1A), and found good retention of morphological characteristics of the original tumors, especially differentiation status, which was also confirmed in lung cancer (Figure S1A and Table S5). We also performed whole-exome sequencing to monitor genomic alterations in 2 CRC CTOS during passages (Figure 1B). Although each line showed a different number of mutations, all CTOS lines showed only a marginal increase in mutation during *in vivo* passages, which was also confirmed in 2 CTOS of lung cancer (Figure S1B). Overall, our observations verified that CTOS lines met the quality requirements for use as a platform for HT screening and evaluation of diversity.

### 3.2 | High-throughput screening of 2427 drugs on 2 colorectal cancer tissue-originated spheroid lines

Unlike established cell lines, from which homogenous spheroids can be prepared, CTOS are heterogeneous in terms of shape and size. It is practically impossible to manually select and dispense CTOS within a specific range of size and quality into each well to test thousands of drugs. Thus, we used an automatic system to select CTOS meeting

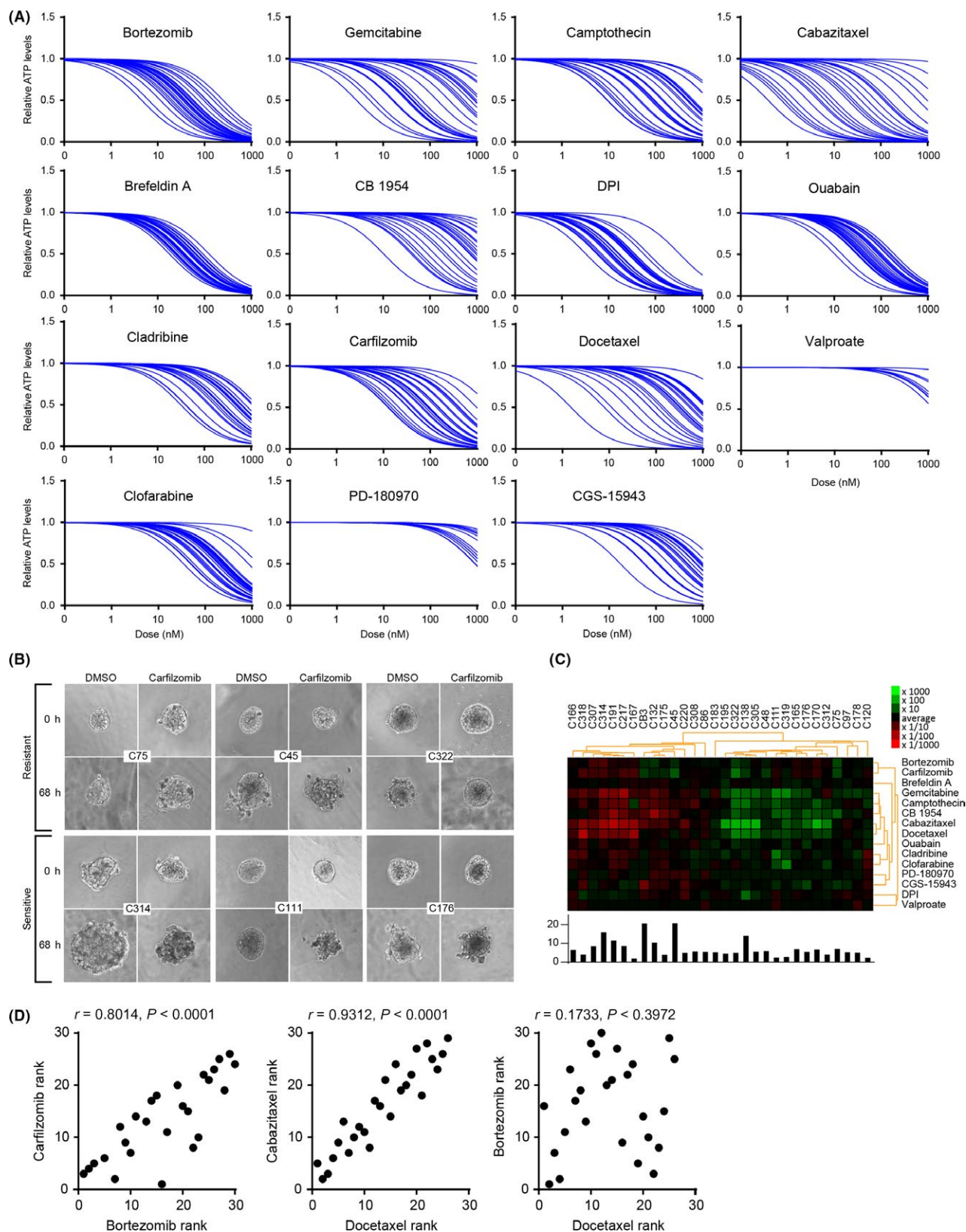
morphological criteria, and to dispense them individually. CTOS were selected based on size (70–100  $\mu$ m) to minimize the effect of hypoxia after growth and on appearance (transparency) to exclude the clusters of dead cells (Figure 2A). Two CRC lines were used for high-throughput screening: the fast-growing C45 line and the slow-growing C132 line. Figures 2B,C show the variations of CTOS size and growth. We screened a total of 2427 drugs (Table S4) at relatively low doses (10 and 100 nmol/L) for their ability to suppress cancer cell growth (Figure 2D). Of these 2427 drugs, we identified 15 (.6%) hit drugs that inhibited CTOS viability down to 40% of control (Table 1), of which 8 drugs were effective against both lines, 2 drugs against C45 alone, and 5 drugs against C132 alone; 10 drugs were anti-cancer reagents and 7 were FDA-approved.

### 3.3 | Hit drug evaluation using a panel of 30 colorectal cancer tissue-originated spheroid

To evaluate the diversity of sensitivity among the 15 hit drugs in various CTOS lines, we used a CRC CTOS panel comprising 30 lines (Tables S1, S2). We had a 100% success rate (30/30) for making xenograft tumors from freeze-stock samples. The CTOS yield from xenograft tumors ranged from 2133 to 331 733 CTOS per gram of tissue, with a variable CTOS size distribution among the preparations (Figures 3A,B). The numbers of CTOS prepared from the xenograft tumors fulfilled the required cell amounts for testing multiple drugs. The basal CTOS growth also varied among lines (Figure 3C).

We analyzed the dose-response curves for the 15 hit compounds tested in 30 CTOS lines, and observed substantial variation in the sensitivity to each compound, which differed among the compounds (Figures 4A, S2). The drugs valproate and PD-180970 were ineffective against all tested lines, including the C45 and C132 lines used for screening, indicating a 13% (2/15) false-positive rate from the high-throughput screening. For the other drugs, the results in C45 CTOS confirmed the test's reproducibility (Figure S3). Morphological changes of the spheroids after drug treatment were not indicative for the drugs' effect (Figure 4B). Clustering analyses were performed based on the patterns of sensitivity towards the 15 drugs (Figure 4C). Based on the clustering analysis of the lines, CTOS lines were separated into 2 groups: CTOS lines that generally exhibited good responses to the examined drugs (left side) and CTOS lines that generally exhibited poor responses (right side). However, this does not necessarily mean that CTOS lines clustered on the right side are merely multi-drug resistant. There are drugs that worked better in CTOS on the right side, and less efficiently in CTOS on the left, indicating the complexed nature of drug sensitivity. In addition, these 2 groups of different drug sensitivity are not a mere reflection of the difference in the basal growth of each CTOS lines (Figure 4C, bottom). Based on the clustering analysis of the drugs, 2 pairs of drugs (the proteasome inhibitors bortezomib and carfilzomib, and the taxanes docetaxel and cabazitaxel) were tightly clustered. We then ranked the sensitivity of 30 individual CTOS lines towards these 4 drugs (Figure 4D). Pairs of drugs with the same mode of action exhibited significant correlation (Figure 4D left and middle panels), while drugs with different mode of action showed no





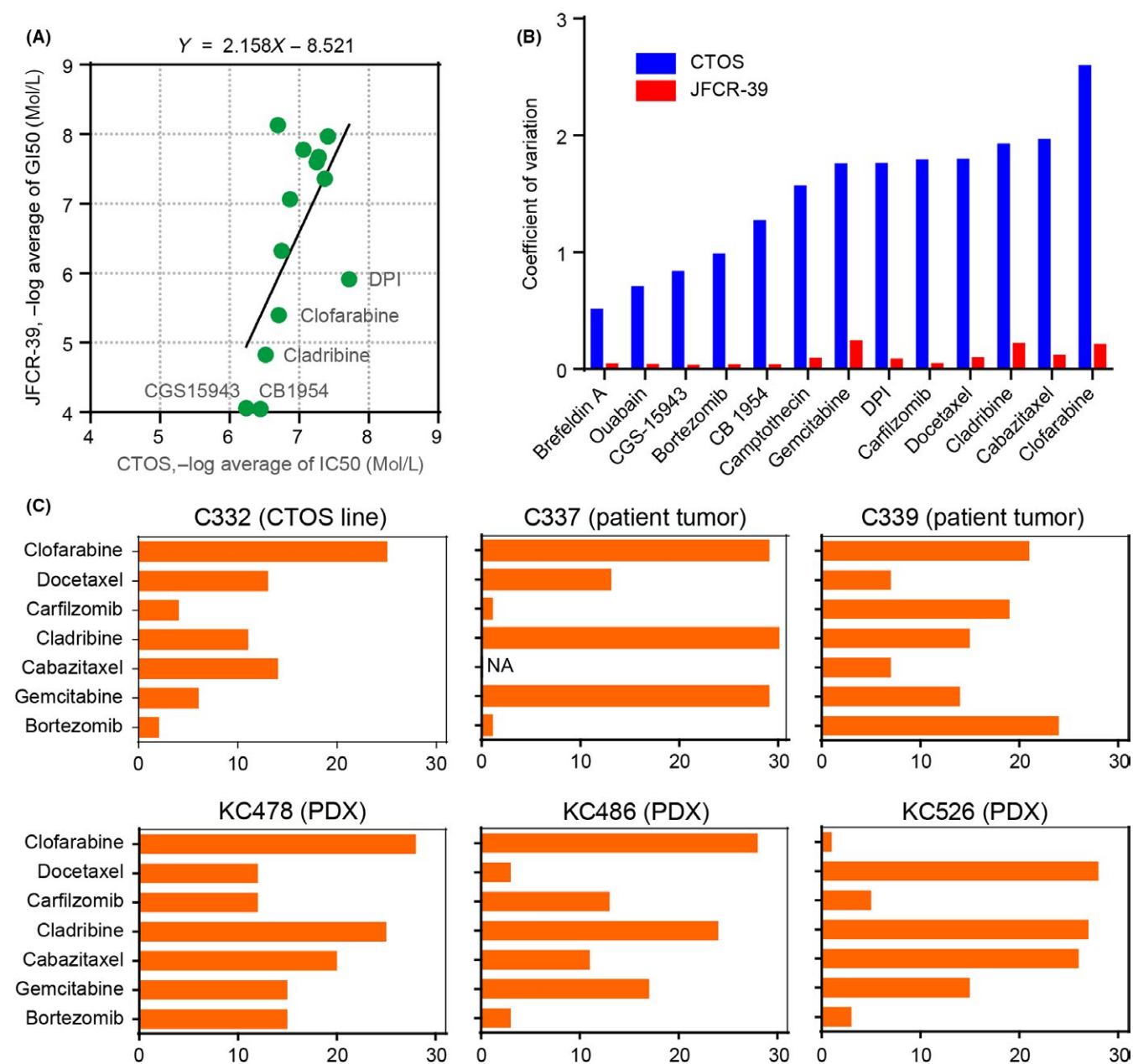
**FIGURE 4** Diversity of sensitivities for the hit drugs among colorectal cancer (CRC) cancer tissue-originated spheroid (CTOS) lines. A, Dose-response curves of 15 drugs in the panel of CRC CTOS lines. B, Morphological changes after treatment with 100 nmol/L of carfilzomib. C, Heat map and clustering analysis of the average IC<sub>50</sub> of 15 drugs in the CTOS panel. The heat map is shown as the  $-\log$  of IC<sub>50</sub> subtracted from the average for each drug. Bar graph shows the mean growth of each CTOS line at basal condition, which is extracted from Figure 3C. D, Ranks of the sensitivity of CTOS lines toward bortezomib and carfilzomib (left), docetaxel and cabazitaxel (middle), and docetaxel and bortezomib (right) are presented in diagrams.  $R$ -value and  $P$ -value were analyzed by Spearman's rank correlation

correlation (Figure 4D, right panel). These data indicate that the assay correctly reflected the mode of action.

### 3.4 | Diversity of sensitivity among cancer tissue-originated spheroids from the colorectal cancer panel

We next compared our results in CRC CTOS with the findings of JFCR39, which is a panel of 39 human cancer cell lines coupled with an NCI60-based drug-activity database.<sup>22,23</sup> We generated a

log-log scatter plot graph by plotting the average IC<sub>50</sub> values (–log) for the 15 drugs in the CRC CTOS panel on the horizontal axis, and the average GI<sub>50</sub> values (–log) in monolayer JFCR39 cell lines on the vertical axis (Figure 5A). Although different methods and different parameters were used, these average values showed a good correlation ( $r^2 = .4076$ ). Five drugs (diphenyleneiodonium, clofarabine, cladribine, CB1954 and CGS015943) exhibited a greater than 30-fold efficacy against CTOS. In contrast, comparing the coefficient of variation revealed that the degree of variation between CTOS lines



**FIGURE 5** Variation of drug sensitivities in the cancer tissue-originated spheroid (CTOS) panel. A, Correlation between the average IC<sub>50</sub> (–log) in the CTOS panel and the average GI<sub>50</sub> (–log) in 2D cultured JFCR39. The drugs that showed higher sensitivity in CTOS lines than JFCR39 lines are named, and the linear regression equation is shown. B, Coefficient of variation of IC<sub>50</sub> in CTOS lines and GI<sub>50</sub> in JFCR39 lines for each drug. C, Ranking of the 7 FDA-approved hit drugs among the 30 reference CTOS lines for the indicated CTOS: C332 (a CTOS line not included in the 30 reference lines); C337 and C339 (primary CTOS prepared directly from patient tumors); and KC478, KC486 and KC526 (PDX-derived CTOS)

in CTOS assay was strikingly higher than that between cancer cell lines in JFCR assay for all examined candidate drugs (Figure 5B). This result supports that the CTOS panel is a suitable platform for studying the extent of response heterogeneity, although the reason of the difference might be due to the different methods used, IC50 and GI50. We further evaluated the sensitivity of each CTOS line to different drugs. We ranked the sensitivity of 30 individual lines towards each FDA-approved drug (Figure S4). The ranking profile differed for individual lines (Figure 5C).

## 4 | DISCUSSION

In most reports of high-throughput screening in 3D cultured cells, spheroids have been generated as the aggregates of established cell lines.<sup>4</sup> Organoid methods have previously been used for smaller scale screenings of 16–160 drugs.<sup>24–26</sup> To our knowledge, this is the first report to describe high-throughput screening of over 2000 compounds in a 3D culture. The present CTOS method enabled the preparation of a large number of CTOS from xenograft tumors (Figure 3A), requiring only several hours for the preparation of cancer cells from tumor tissue, and enabling overnight CTOS formation. Moreover, the cells comprising the CTOS showed high viability and stability compared to the primary cells dissociated into single cells.<sup>8,27</sup> These advantages of CTOS enabled the high throughput screening using 3D and ex vivo culture.

The CTOS method supports ex vivo applications of mouse xenografts, including high-throughput screening, as in our present study, genetic manipulation of cancer cells,<sup>10,11</sup> and detailed analysis of intracellular signaling in response to various stimuli.<sup>9,12</sup> Notably, CTOS can be freeze-stocked with an excellent recovery rate, helping to avoid over-passage of patient-derived xenografts. Here we demonstrated that a CTOS ex vivo assay can also be applied to PDX (Figure 5C). It is costly and time-consuming to expand CTOS in vitro. However, large numbers of CTOS can be prepared from a xenograft tumor within approximately 1 month (Figure 3A). Thus, the CTOS method complements mouse xenograft methods. We demonstrated that some genomic changes occurred in CTOS throughout 5 passages via mouse xenografts (Figures 1B, S1B), such as passaged tumors of PDX.<sup>28</sup> One should keep in mind this slight genomic changes might affect the results of sensitivity assay and can be the cause for a discrepant result with patient's clinical response, when clinical application is the subject of a study.

Our present results showed that drug sensitivity substantially varied among the CTOS lines. This variation of sensitivity is relevant to 2 different aspects of application. First, as shown in this study, sensitivity ranking enables assessment of the sensitivity of an individual CTOS relative to a reference panel of CTOS lines (Figure 5C). Using more lines as the reference would increase the accuracy of the prediction. This method might be useful in the selection of drugs for individual patients in future, especially when the drugs are tested in clinical trials that indicate the variable sensitivity among patients. For this application, technical improvements

in the preparation of CTOS from patient tumor biopsy samples is critical to test multiple drugs for each patient. Although CTOS lines did not change significantly over several passages in terms of histology and mutations, the quality of CTOS for the purpose of personalized drug selection should be carefully considered: for example, a possible single mutation over ex vivo passage could change the drug sensitivity. Second, classifying CTOS lines into sensitive and resistant groups with large collections of PDX and organoids might help in biomarker identification.

Because a CTOS comprises pure cancer cells,<sup>8</sup> the CTOS sensitivity assay in our study would not fully reflect the complex tumor microenvironment. Non-physiological oxygen concentration may be another issue. Nonetheless, it provides important information because cancer cell sensitivity per se is one of the critical factors in a patient's drug sensitivity. For some of the drugs used in clinic, CTOS sensitivity assay results were consistent with the reported results of clinical trials specifically in the cases of an EGFR tyrosine kinase inhibitor in lung cancer<sup>9</sup> and an anti-EGFR antibody in colorectal cancer.<sup>12</sup> However, the clinical relevance of the CTOS sensitivity assay should be further assessed in prospective clinical trials, such as a recent study that compared the drug responses of patient-derived gastrointestinal organoids with the responses of the patients in the clinic.<sup>29</sup> It may be necessary to optimize the culture conditions for each drug<sup>9,12</sup> to better reflect the clinical responses of individual patients to the drug.

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## CONFLICT OF INTEREST

M.I. is an inventor of a patent related to this work. J.K. and M.I. currently belong to the Department of Clinical Bio-resource Research and Development, which is sponsored by KBBM.

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## REFERENCES

1. Aberle MR, Burkhart RA, Tiriach H, et al. Patient-derived organoid models help define personalized management of gastrointestinal cancer. *Br J Surg*. 2018;105:e48–e60.
2. Letai A. Functional precision cancer medicine-moving beyond pure genomics. *Nat Med*. 2017;23:1028–1035.
3. Witkiewicz AK, Balaji U, Eslinger C, et al. Integrated patient-derived models delineate individualized therapeutic vulnerabilities of pancreatic cancer. *Cell Rep*. 2016;16:2017–2031.
4. Friedrich J, Seidel C, Ebner R, Kunz-Schughart LA. Spheroid-based drug screen: considerations and practical approach. *Nat Protoc*. 2009;4:309–324.
5. Horvath P, Aulner N, Bickle M, et al. Screening out irrelevant cell-based models of disease. *Nat Rev Drug Discov*. 2016;15:751–769.

6. Drost J, Clevers H. Organoids in cancer research. *Nat Rev Cancer*. 2018;18:407-418.
7. Sachs N, Clevers H. Organoid cultures for the analysis of cancer phenotypes. *Curr Opin Genet Dev*. 2014;24:68-73.
8. Kondo J, Endo H, Okuyama H, et al. Retaining cell-cell contact enables preparation and culture of spheroids composed of pure primary cancer cells from colorectal cancer. *Proc Natl Acad Sci USA*. 2011;108:6235-6240.
9. Endo H, Okami J, Okuyama H, et al. Spheroid culture of primary lung cancer cells with neuregulin 1/HER3 pathway activation. *J Thorac Oncol*. 2013;8:131-139.
10. Endo H, Okami J, Okuyama H, Nishizawa Y, Imamura F, Inoue M. The induction of MIG6 under hypoxic conditions is critical for dormancy in primary cultured lung cancer cells with activating EGFR mutations. *Oncogene*. 2016;36:2824-2834.
11. Nakajima A, Endo H, Okuyama H, et al. Radiation sensitivity assay with a panel of patient-derived spheroids of small cell carcinoma of the cervix. *Int J Cancer*. 2015;136:2949-2960.
12. Tashiro T, Okuyama H, Endo H, et al. In vivo and ex vivo cetuximab sensitivity assay using three-dimensional primary culture system to stratify KRAS mutant colorectal cancer. *PLoS ONE*. 2017;12:e0174151.
13. Kiyohara Y, Yoshino K, Kubota S, et al. Drug screening and grouping by sensitivity with a panel of primary cultured cancer spheroids derived from endometrial cancer. *Cancer Sci*. 2016;107:452-460.
14. Yoshii Y, Furukawa T, Waki A, et al. High-throughput screening with nanoimprinting 3D culture for efficient drug development by mimicking the tumor environment. *Biomaterials*. 2015;51:278-289.
15. Taniguchi E, Nakajima H, Hongyo T, et al. Effects of N-methyl-N'-nitro-N-nitrosoguanidine on the human colorectal polyps consecutively maintained in SCID mice. *Cancer Lett*. 2002;182:127-133.
16. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25:1754-1760.
17. DePristo MA, Banks E, Poplin R, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet*. 2011;43:491-498.
18. Koboldt DC, Zhang Q, Larson DE, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res*. 2012;22:568-576.
19. Cibulskis K, Lawrence MS, Carter SL, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol*. 2013;31:213-219.
20. Magi A, Tattini L, Cifola I, et al. EXCAVATOR: detecting copy number variants from whole-exome sequencing data. *Genome Biol*. 2013;14:R120.
21. Monks A, Scudiero D, Skehan P, et al. Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines. *J Natl Cancer Inst*. 1991;83:757-766.
22. Yamori T, Matsunaga A, Sato S, et al. Potent antitumor activity of MS-247, a novel DNA minor groove binder, evaluated by an in vitro and in vivo human cancer cell line panel. *Cancer Res*. 1999;59:4042-4049.
23. Shoemaker RH. The NCI60 human tumour cell line anticancer drug screen. *Nat Rev Cancer*. 2006;6:813-823.
24. Pauli C, Hopkins BD, Prandi D, et al. Personalized in vitro and in vivo cancer models to guide precision medicine. *Cancer Discov*. 2017;7:462-477.
25. Schutte M, Risch T, Abdavi-Azar N, et al. Molecular dissection of colorectal cancer in pre-clinical models identifies biomarkers predicting sensitivity to EGFR inhibitors. *Nat Commun*. 2017;8:14262.
26. van de Wetering M, Francies HE, Francis JM, et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell*. 2015;161:933-945.
27. Yoshida T, Okuyama H, Nakayama M, et al. High-dose chemotherapeutics of intravesical chemotherapy rapidly induce mitochondrial dysfunction in bladder cancer-derived spheroids. *Cancer Sci*. 2015;106:69-77.
28. Ben-David U, Ha G, Tseng YY, et al. Patient-derived xenografts undergo mouse-specific tumor evolution. *Nat Genet*. 2017;49:1567-1575.
29. Vlachogiannis G, Hedayat S, Vatsiou A, et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science*. 2018;359:920-926.

## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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